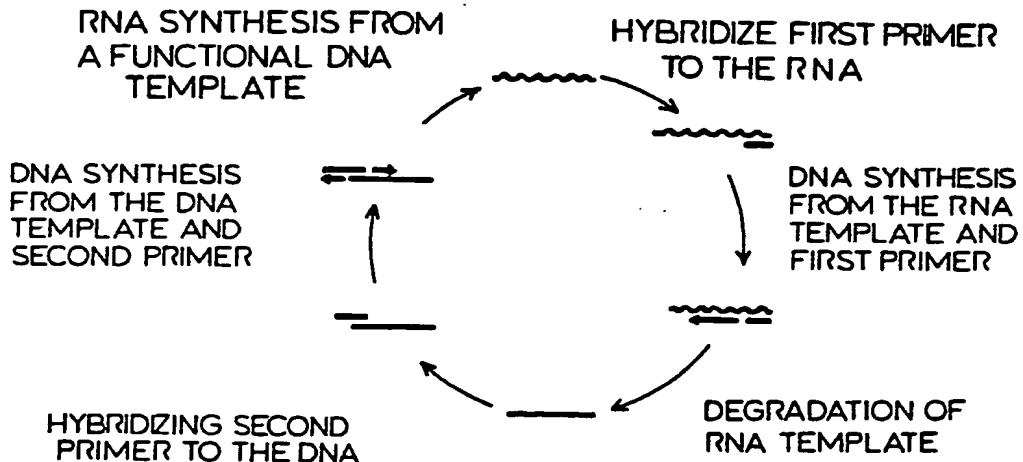




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## (54) Title: NUCLEIC ACID AMPLIFICATION PROCESS



## (57) Abstract

This invention relates to a process for amplifying a specific nucleic acid sequence. The process involves synthesizing single-stranded RNA, single-stranded DNA and double-stranded DNA. The single-stranded RNA is a first template for a first primer, the single-stranded DNA is a second template for a second primer, and the double-stranded DNA is a third template for synthesis of a plurality of copies of the first template. A sequence of the first primer or the second primer is complementary to a sequence of the specific nucleic acid and a sequence of the first primer or the second primer is homologous to a sequence of the specific nucleic acid. The amplification process may be used to increase the quantity of the specific nucleic acid sequence to allow detection, or to increase the purity of the specific nucleic acid sequence as a substitute for conventional cloning methodology.

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## NUCLEIC ACID AMPLIFICATION PROCESS

FIELD OF THE INVENTION

This invention relates to a process for amplifying a specific nucleic acid sequence.

BACKGROUND OF THE INVENTION

The detection of a specific nucleic acid sequence present in a sample by probing the sample with a complementary sequence of nucleic acids is a known diagnostic technique. Nucleic acids are highly specific in binding to complementary nucleic acids and are thus useful to determine whether a specific nucleic acid is present in a sample. One must know the sequence of the specific nucleic acid to be detected and then construct a probe having a complementary nucleic acid sequence to the specific nucleic acid sequence.

In this application, the phrase "specific nucleic acid sequence" means a single stranded or double

stranded nucleic acid which one wishes to amplify; "sample" means a mixture containing nucleic acids; "sufficiently complementary" means that two nucleic acids, a primer and a template, are capable of specific interaction which allows efficient, primer-dependent and template-directed synthesis of DNA, under given conditions of ionic strength and temperature.

Since nucleic acid probes are highly specific, it is preferable in some situations to probe the nucleic acid sequence itself rather than the protein produced by the nucleic acid sequence. As a particular example, a diagnostic method based solely on protein detection would be unreliable for determining the presence of infectious particles of hepatitis B virus, due to the presence of significant levels of non-infectious antigen particles which lack the DNA genome. In another example, the various subtypes of human papilloma virus found in either pre-cancerous or benign cervical tumors can be distinguished only by the use of nucleic acid probe hybridization. Also, the microbiology of AIDS makes it certain that an assay based on the presence of AIDS specific nucleic acid sequence would be superior as a diagnostic.

The greatest difficulty with applying existing nucleic acid probe technology, and the reason that the utility of the existing probe technology is limited, is the copy number problem. In a virus or cell, for example, there is usually a single copy of a particular gene. This one copy may give rise to many copies of gene product, either RNA or protein. For this reason, diagnostic techniques have often involved probing the protein, since the specific sequence of nucleic acid to be detected may give rise to many thousand copies of protein.

The naturally-occurring high number of ribosomal RNA, up to 100,000 copies per cell, has been used by GenProbe to facilitate diagnosis of certain bacterial pathogens, such as Legionella and Mycoplasma, using nucleic acid probes. However, this strategy cannot be used with non-cellular pathogens, such as viruses. Copy number is a particular problem with the development of a nucleic acid probe method for the detection of AIDS virus, where the integrated provirus may be present in less than one of ten thousand peripheral blood lymphocytes. Thus, if the particular nucleic acid sequence suspected to be present in a sample could be amplified, the copy number problem could be circumvented and probe assays could be more readily used.

In a normal biological sample, containing only a few cells, and consequently only a few copies of a particular gene, it is necessary to utilize an amplification process in order to overcome the copy number problem.

One method to amplify is to 'grow out' the sample, that is, to arrange conditions so that the living biological material present in the sample can replicate itself. Replication increases the quantity of nucleic acid sequences to detectable levels. In the food industry, for example, in order to test processed food for the food-poisoning bacteria *Salmonella*, food samples must be incubated for a number of days to increase the quantity of nucleic acids. In clinical samples, pathogens must also be allowed to increase their number by growing out over some considerable time.

United States Patent No. 4,683,195 issued on July 28, 1987 to Cetus Corporation and United States Patent No. 4,683,202 issued on July 28, 1987 to Cetus Corporation are each directed to a process for amplifying a target nucleic acid sequence contained in a sample. United States Patent No. 4,683,195 relates

to a process in which a sample suspected of containing a target nucleic acid sequence is treated with oligonucleotide primers such that a primer extension product is synthesized which in turn serves as a template, resulting in amplification of the target nucleic acid sequence. The primer extension product is separated from the template in the preferred embodiment using heat denaturation. Similarly, United States Patent No. 4,683,202 relates to a process for amplifying a target nucleic acid sequence having two separate complementary strands. The process includes treating the strands with primers to synthesize extension products, separating the primer extension products from the templates, and in turn using the primer extension products as templates.

Both of the above United States patents require either manual or mechanical participation and multi-step operations by the user in the amplification process. The steps involved in these patents require the user to heat the sample, cool the sample, add appropriate enzymes and then repeat the steps. The temperature changes cause the enzymes to lose their activity. Hence, the user is required to repeatedly supplement the amplification mixture with aliquots of appropriate enzymes during the amplification process.

In addition, in United States Patents Nos. 4,683,195 and 4,683,202 each cycle of the amplification process takes place by the synthesis from a first template, of a second template, the second template in turn is used to synthesize the first template. This procedure is repeated, thus, each cycle of the amplification process is based on the synthesis of one product from one substrate.

Notwithstanding the amplification processes disclosed in the prior art, a need exists for improvements to the amplification process. It would be preferable if the amplification process required less participation and fewer manipulations by the user. Further, it would be advantageous if the amplification took place at a relatively constant ambient temperature so that the activity of the enzymes involved in the process would not be affected. It would be more expedient if a template could be used to generate more than one product from one substrate in each cycle of the amplification process.

SUMMARY OF THE INVENTION

This invention relates to an amplification process which is expedient and requires less participation and fewer manipulations by the user of the process than conventional amplification processes. The amplification takes place at a relatively constant ambient temperature. In addition, each cycle of the process generates a plurality of copies of product from one substrate. The amplification process of this invention may be used to increase the quantity of a specific nucleic acid thus circumventing the copy number problem. Hence, probe assays may be more readily used. The amplification process could also be used to increase the purity of a specific nucleic acid sequence as a substitute for conventional cloning methodology.

According to one aspect of the invention, a process for amplifying a specific nucleic acid sequence is used. The process involves the synthesis of single-stranded RNA, single-stranded DNA, and double stranded DNA. The single stranded RNA is a first template for a first primer. The single stranded DNA is a second template for a second

primer. The double stranded DNA is a third template for the synthesis of a plurality of copies of the first template. A sequence of the first or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence and a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence. A 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

According to another aspect of the invention, a process for amplifying a specific nucleic acid sequence is used. The process involves:

- (a) hybridizing a first primer to a first template. The first primer has a DNA sequence which is sufficiently complementary to a RNA sequence of the first template;
- (b) synthesizing a first DNA sequence covalently attached to the first primer and complementary to the RNA sequence of the first template. The first DNA sequence and the first primer comprise a second template;

- (c) separating the first template from the second template to allow hybridization of a second primer;
- (d) hybridizing the second primer to the second template. The second primer has a DNA sequence which is sufficiently complementary to a DNA sequence of the second template. The second primer also has a sequence of a promoter and a sequence of a transcription initiation site for a RNA polymerase;
- (e) synthesizing a second DNA sequence covalently attached to the second primer and complementary to the DNA sequence of the second template and synthesizing a third DNA sequence covalently attached to the second template and complementary to the DNA sequence of the second primer. The second and third DNA sequences, the second primer and the second template comprise a third template;

(f) synthesizing a plurality of copies of the RNA sequence of the first template from the third template.

A sequence of the first or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence and a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence. A 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

In a further alternative of the invention, the second primer of DNA has a sequence at its 3' end which is sufficiently complementary to the DNA sequence of the second template. The second primer has at its 5' end a sequence of a promoter and a sequence of a transcription initiation site for a RNA polymerase.

In another alternative of the invention, a process for amplifying a specific nucleic acid sequence is used. The process involves combining a first primer, a second primer, ribonuclease H, a RNA-directed DNA polymerase, a DNA-directed DNA polymerase, a RNA polymerase, ribonucleoside triphosphates and deoxyribonucleoside triphosphates with a sample. The first primer of DNA has a sequence which is sufficiently complementary to a first template of RNA. The second primer of DNA has a sequence which is sufficiently complementary to a second template of DNA, and a sequence of a promoter and a sequence of a transcription initiation site which are recognized as substrate by the RNA polymerase. A sequence of the first primer or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence and a sequence of the first primer or the second primer is sufficiently homologous to a sequence of the specific nucleic acid. A 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

In a further alternative of the invention, a process for amplifying a specific nucleic acid sequence is used. The process involves adding a first

primer, a second primer, avian myoblastosis viral polymerase, E. coli ribonuclease H, bacteriophage T7 RNA polymerase, ribonucleoside triphosphates and deoxyribonucleoside triphosphates to a sample. The first primer of DNA has a sequence which is sufficiently complementary to a first template of RNA. The second primer of DNA has a sequence which is sufficiently complementary to a second template of DNA, and a sequence of a promoter and a sequence of a transcription initiation site which are recognized as substrate by T7 RNA polymerase. A sequence of the first primer or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence and a sequence of the first primer or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence. A 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

#### BRIEF DESCRIPTION OF THE DRAWINGS

In drawings which illustrate embodiments of the invention,

**Figure 1** is a general illustration the nucleic acid amplification process;

**Figure 2** shows the synthetic oligonucleotides DNA sequences which are used for testing the amplification process:  
**Figure 2A**, the gag test sequence;  
**Figure 2B**, the gag2 test sequence;

**Figure 3** is an autoradiogram of PAGE analysis of amplification reactions using different primer concentrations;

**Figure 4** is an autoradiogram of PAGE analysis of amplification reactions using different template concentrations;

**Figure 5** is an autoradiogram of Dot-blot hybridization on amplification reactions;

**Figure 6** is an autoradiogram of PAGE analysis of amplification reaction using restriction fragments as template;

Figure 7 is a general illustration of the indirect nucleic acid amplification process.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention relates to a process for amplifying a specific nucleic acid sequence. The amplification involves the alternate synthesis of DNA and RNA and is generally illustrated in Figure 1. In this process, single-stranded RNA is converted to single-stranded DNA which in turn is converted to a functional template for the synthesis of a plurality of copies of the original single-stranded RNA. A first primer and a second primer are used in the amplification process. A sequence of the first primer or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence and a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence. In some instances, both the first primer and second primer are sufficiently complementary and sufficiently homologous to a sequence of the specific nucleic acid sequence, for example, if the specific nucleic acid sequence is double stranded DNA.

The RNA is converted to single-stranded DNA by hybridizing an oligonucleotide primer (the first

primer) to the RNA (the first template) and synthesizing a complementary strand of DNA from the first primer, (the first DNA sequence) by using a RNA-directed DNA polymerase. The resulting single-stranded DNA (the second template) is separated from the first template by, for example, hydrolysis of the first template and by using a ribonuclease which is specific for RNA-DNA hybrids (for example, ribonuclease H). The second template is converted to a form which is capable of RNA synthesis by hybridizing a synthetic oligonucleotide (the second primer), which contains at its 3' end a sequence which is sufficiently complementary to the 3' end of the second template and toward its 5' end a sequence of a promoter and a sequence of a transcription initiation site, and by synthesizing a second DNA sequence covalently attached to the 3' end of the second primer using the second template as a template and synthesizing a third DNA sequence covalently attached to the 3' end of the second template using the second primer as a template, using DNA-directed DNA polymerase. The resulting functional derivative of the second template, which is a third template, is used for the synthesis of a plurality of copies of RNA, the first template, by

using a RNA polymerase which is specific for the promoter and transcription initiation site defined by the second primer. Each newly synthesized first template can be converted to further copies of the second template and the third template by repeating the cycle. In addition, repetition of the cycle does not require participation or manipulation by the user.

The amplification process commences with the addition of a suitable template nucleic acid to the appropriate enzymes, primers, and cofactors under the appropriate reaction conditions. This template nucleic acid is in a form which is capable of homogenous and continuous amplification and can function as an intermediate in the cycle set forth in Figure 1. The amplification process involves the net consumption of precursors (primers, ribonucleoside triphosphates and deoxyribonucleoside triphosphates) and the net accumulation of products (RNA and DNA). The processes of RNA and DNA synthesis will proceed asynchronously until sufficient levels of nucleic acids have been synthesized to allow detection. The amplification process may be monitored by, for example, the synthesis of a labeled product from a labeled precursor.

It is contemplated that amplification may involve another process either in addition to or in place of the one generally illustrated in Figure 1. Also possible are certain counter-productive enzymatic reactions which occur at permissibly low rates. Included among the possible non-productive side reactions is the synthesis of RNA and/or DNA in the absence of an added template nucleic acid. Such RNA and/or DNA products can be discriminated from desired products by determining whether a particular sequence which would be found only between the two priming sites of the specific nucleic acid sequence is present.

The first primer is an oligodeoxyribonucleotide which has at its 3' end a sequence which is sufficiently complementary to the 3' end of the first template. The sequence at the 3' end of the first primer has a particular length and base composition to allow specific and efficient synthesis of the first DNA sequence, under the given conditions of ionic strength and temperature. The first primer may be sufficiently complementary to a region internal to the 3' end of the first template in the first cycle. In subsequent cycles, the 5' end of the first primer would be complementary to the 3' end of the first

template. It is contemplated that the first primer may be composed partially or completely of nucleotides or nucleotide analogs other than the natural deoxyribonucleotides. The 5' end of the first primer may contain sequences which are not complementary to the first template in the first cycle. The non-complementary sequences may be complementary to a nucleic acid which can be immobilized, or to which can be bound a useful non-nucleic acid component, such as a reporter to facilitate detection.

The second primer is an oligodeoxyribonucleotide which contains at its 3' end a sequence which is sufficiently complementary to the 3' end of the second template. The second primer has a particular length and base composition to allow specific and efficient synthesis of the second and third DNA sequences, under the given conditions of ionic strength and

temperature. In addition, the second primer contains the sequence of a functional promoter and the sequence of a transcription initiation site. This sequence, when used as a template for synthesis of the third DNA sequence, contains sufficient information to allow specific and efficient binding of a RNA polymerase and initiation of transcription at the desired site. The promoter sequence may be derived from the antisense strand of a functional promoter. The transcription initiation site may be derived from the 5'-terminal sequence of a natural RNA transcript. In the preferred embodiment, the 5'-terminal sequence of the second primer is AATTCTAATACGACTCACTATAGGGAG. This sequence contains the sequence of the promoter and the sequence of the transcription initiation site for T7 RNA polymerase. Alternatively, the transcription initiation site and promoter for another phage RNA polymerase may be used. In addition, sequences which are unrelated to the promoter function may be included at the 5' end of the second primer or between the transcription initiation site and the sequence at the 3' end which hybridizes to the second template. It is contemplated that the second primer may be composed partially or completely of nucleotides or nucleotide analogs other than natural deoxyribonucleotides.

All of the enzymes used in this invention should meet certain practical specifications. Each enzyme or enzyme preparation should be free of deleterious deoxyribonuclease ("DNase") activities, such as the 5' or 3' exonuclease activities which are often associated with certain DNA polymerases and single-strand or double-strand specific exonuclease or endonucleases. Each enzyme or enzyme preparation should be free of deleterious ribonuclease ("RNase") activities, with the exception of the preferred addition of a ribonuclease activity which is specific for hybrids of RNA and DNA (for example, ribonuclease H). In addition, each enzyme should be reasonably active under the common reaction conditions which are used for the other enzymatic processes, and non-enzymatic processes, such as hybridizing oligonucleotide primers to the RNA or DNA templates.

The DNA-directed RNA polymerase which is used in this invention may be any enzyme capable of binding to a particular DNA sequence called a promoter and specifically initiating in vitro RNA synthesis at a defined initiation site within close proximity to the promoter. The promoter and the initiation site form part of the second primer. In addition the RNA

polymerase should be capable of synthesizing several copies of RNA per functional copy of template in a reasonable amount of time. In the preferred embodiment, the bacteriophage T7 RNA polymerase is used. In addition other bacteriophage RNA polymerases, such as phage T3, phage  $\phi$ II, salmonella phage sp6<sup>-</sup>, or pseudomonas phage gn-1 may be used. In another embodiment, other prokaryotic or eukaryotic DNA-directed RNA polymerase may be used. It should be understood that if alternative RNA polymerases are used, then the necessary changes to the promoter and initiation sequences of the second primer should be made according to the template specificity of the particular RNA polymerase.

The RNA-directed DNA polymerase which is used in this invention may be any enzyme capable of synthesizing DNA from an oligodeoxyribonucleotide primer and a RNA template. In addition this enzyme may contain activities for DNA-directed DNA polymerase and RNase H. In the preferred embodiment, the avian myoblastosis viral polymerase ("AMV reverse transcriptase") is used. In addition, the RNA-directed DNA polymerase could be from another retrovirus, such a Maloney murine leukemia virus.

Alternatively, other eukaryotic RNA-directed DNA polymerases could be used.

The DNA-directed DNA polymerase which is used in this invention may be any enzyme capable of synthesizing DNA from an oligodeoxyribonucleotide primer and a DNA template. This enzyme should not contain either 5'- or 3'-exonuclease activities, which are associated with many types of DNA polymerase. In the preferred embodiment, the AMV reverse transcriptase is used. However, other DNA-directed DNA polymerase which naturally lack the 5'- or 3'-exonuclease activities could be used. These could include certain eukaryotic DNA polymerases, such as, DNA polymerase  $\alpha$  or  $\beta$  those DNA polymerases which could be isolated from a mammalian tissue, such as calf thymus. An otherwise unsuitable DNA polymerase could be made useful by removing the undesirable exonuclease activities either by alteration of the DNA polymerase gene followed by expression of the altered polymerase in a suitable host cell, or by chemical modification of the DNA polymerase protein. Altered versions of DNA polymerase could be made from the Klenow fragment of *E. coli* DNA polymerase I or the bacteriophage T7 DNA

polymerase. It should be understood that such alternative DNA-directed DNA polymerase activities are added to supplement the activity contributed by the RNA-directed DNA polymerase, since in the preferred embodiment, both RNA-directed and DNA-directed DNA polymerase activities are supplied by the same enzyme.

The RNase H which could be used in this invention may be any enzyme capable of hydrolyzing a RNA which is annealed to a complementary DNA. This enzyme should not be capable of hydrolyzing single or double-stranded RNA or any DNA. In the preferred embodiment, the *E. coli* RNase H is used. In addition, other RNase H enzymes could be used, such as calf thymus RNase H. Since RNase H is an intrinsic activity of AMV reverse transcriptase, the *E. coli* RNase H will be supplemented in the preferred embodiment by the RNase H of AMV reverse transcriptase. Alternatively, any other enzyme capable of separating the second template from the first template could be used.

The abovementioned enzymes and primers are mixed together in a reaction vessel which contains the necessary buffers and cofactors for both DNA and RNA

synthesis. In addition, the ionic conditions and reaction temperature should be compatible with specific hybridization of the primers to the DNA and RNA templates as is known to those skilled in the art. The reaction mixture should be free of such agents which would interfere with the amplification process, specifically substances which could greatly inhibit the activity of the enzymes, interfere with the hybridizing of primers and templates, or degrade non-productively the nucleic acid intermediates and products.

The description of possible detection schemes may be useful to the application of the amplification process. It should be understood that schemes which may be used for detecting the nucleic acids which are synthesized in the amplification process are not limited to those described herein, and it is contemplated that other methods may be used.

In one embodiment, a labeled precursor may be added to the reaction mixture. Amplification is determined by quantitative or qualitative analysis of labeled products, which can be separated from the labeled precursor by using methods known in the art.

A labeled precursor may be a ribonucleoside triphosphate for detecting RNA synthesis, or a deoxynucleoside triphosphate or an oligonucleotide primer for detecting DNA synthesis. The type of label may be a radioisotope or a useful chemical group, such as biotin, a chromophore, a fluorophore, or a hapten which could bind to an antibody, or possibly a protein or an enzyme. The labeled products may be separated from the labeled precursors on the basis of solubility, charge, or size. In addition, the labeled DNA or RNA may be hybridized to a nucleic acid which contains a complementary sequence and which can be immobilized.

In another embodiment, the products of the amplification process may be bound to an immobilized support, hybridized to a nucleic acid probe containing a complementary sequence, and separated from the unhybridized nucleic acid probe which remains in solution. The products, DNA or RNA, may be bound directly to a solid support by any stable interaction, such as hydrophobic, electrostatic, or covalent interaction. In addition, the products may contain certain chemical groups, for example, biotin, which may be incorporated into the products during the

amplification process to allow binding to an immobilized protein, for example, avidin or streptavidin. In addition, the products may be hybridized to a nucleic acid which contains a complementary sequence and which can be immobilized. The nucleic acid probe would contain a complementary sequence which forms a sufficiently stable interaction with a product of the amplification process to allow binding under the conditions of hybridization and sustained binding under the conditions used for removal of the unhybridized nucleic acid probe. In the preferred embodiment the complementary sequence would be derived from that part of the specific nucleic acid sequence which is between the sequences of the first primer and the second primer. The nucleic acid probe may be a single-stranded DNA or RNA, or a double-stranded DNA or RNA which can be made single-stranded, or an oligonucleotide which can be composed of deoxyribonucleotides and/or ribonucleotides. In addition, the nucleic acid probe may contain a chemical group which could covalently bind to a product DNA or RNA under the appropriate conditions. The nucleic acid probe may be labeled with a radioisotope or a useful chemical group, such as biotin, a chromophore, a fluorophore, or a hapten.

which could bind to an antibody. In addition, the nucleic acid probe could be conjugated to a protein or enzyme, for example, a phosphatase or a peroxidase. In addition, the nucleic acid probe may contain sequences which would allow in vitro replication of the probe.

It is contemplated that the products of the amplification process may be analyzed by methods which are typically used for nucleic acids that have been enriched by molecular cloning techniques. In one alternative, the synthesis of a specific DNA sequence may be detected by digestion of the synthesized DNA with a restriction endonuclease, followed by electrophoretic separation and detection using methods known in the art. In another alternative, the sequence of amplified RNA may be determined by DNA synthesis using a RNA-directed DNA polymerase, the first primer, and dideoxynucleoside triphosphates (Stoflet et al., 1988). In another alternative, the sequence of the amplified third template may be determined by RNA synthesis using the DNA-directed RNA polymerase used in the amplification process, and 3'-deoxyribonucleoside triphosphates (Axelrod & Kramer, 1985). In another alternative, the amplified

RNA may encode a polypeptide which could be translated, in vitro. The polypeptide product of the in vitro translation could be analyzed by using an antibody.

A sample suspected of containing or known to contain the specific nucleic acid sequence is added to the reaction mixture in the form of a template nucleic acid which is capable of homogeneous and continuous amplification and may be any intermediate in the cycle set forth in Figure 1. In particular, the template nucleic acid may be a single-stranded RNA which contains at its 5' end a sequence which is sufficiently homologous to that which is at the 3' end of the second primer, and contains a sequence which is sufficiently complementary to the first primer. A template nucleic acid of this form would function as a first template in the amplification process. Alternatively, the template nucleic acid may be a single-stranded DNA which contains at its 3' end a sequence which is sufficiently complementary to at least the 3' end of the second primer, and contains a sequence which is sufficiently homologous to that which is at the 3' end of the first primer. A template nucleic acid of this form would function as a

second template in the amplification process. Alternatively, the template nucleic acid may be a double-stranded DNA, one strand of which contains at its 5' end the entire sequence of the second primer and contains a sequence which is sufficiently complementary to the first primer. The double-stranded DNA functions as a third template in the amplification process.

Although the preparation of a template nucleic acid is not part of the amplification process, the description of possible schemes for generating template nucleic acids may be useful to the application of the amplification process. It should be understood that the schemes which may be used for obtaining the template nucleic acid are not limited to the alternatives which are described herein, and it is contemplated that other methods may be used.

In one alternative, a template nucleic acid which could function as a first template could be a naturally occurring RNA or a RNA fragment which could be generated from a larger RNA molecule by using site specific hydrolysis methods known in the art (Shibahara et al., 1987).

In another alternative, a template nucleic acid which could function as a second template could be generated from a double-stranded DNA by digestion with a restriction endonuclease which has a site immediately flanking the sequence which is sufficiently complementary to the 3' end of the second primer. The resulting double-stranded DNA fragments could then be made single-stranded by using chemical or thermal denaturation methods.

In another alternative, a template nucleic acid which could function as a second template could be generated from a single-stranded DNA or RNA to which has been hybridized an oligonucleotide which is capable of blocking DNA synthesis. This blocking oligonucleotide may contain a chemical group, which could covalently bind to the template, under the appropriate conditions. DNA synthesis from this blocked template using the first primer could result in a synthesized DNA with the same 3' end as the second template. If the original template is RNA, then the resulting DNA-RNA hybrid may be used directly as a template nucleic acid. If the original template is DNA, then the resulting copy of the second template could then be separated from the original template by using chemical or thermal denaturation methods.

In another alternative, a template nucleic acid which could function as a third template could be generated from a single-stranded DNA or RNA by DNA synthesis from the DNA or RNA template using the second primer. The resulting synthesized DNA could then be separated from the original template by using chemical or thermal denaturation methods. In addition, a RNA template could be hydrolyzed by using chemical or enzymatic methods. The resulting single-stranded DNA has the sequence of the second primer covalently attached to its 5' end and contains a sequence which is sufficiently complementary to the first primer. This single-stranded DNA could be converted to a transcriptionally functional double-stranded DNA by hybridizing the first primer to the single-stranded DNA, and by synthesizing a DNA sequence which is covalently attached to the first primer and complementary to the single-stranded DNA.

In a further alternative, a single-stranded DNA or RNA template could be obtained from a double-stranded DNA, double-stranded RNA or a DNA-RNA hybrid by using chemical, thermal, or possibly enzymatic methods. Then, by using one of the alternative schemes proposed above, the resulting

single-stranded DNA or RNA could then be used to generate a template nucleic acid which could function as a first, second or third template. In addition, an alternative scheme involving the first primer and one strand of nucleic acid, and another alternative scheme involving the second primer and the other (complementary) strand of the nucleic acid may be used concurrently to generate template nucleic acids.

#### MATERIALS AND METHODS

##### Materials

Oligonucleotides were synthesized using an Applied Biosystems 380A DNA synthesizer. Columns, phosphoramidites, and reagents used for oligonucleotide synthesis were obtained from Applied Biosystems, Inc. through Technical Marketing Associates. Oligonucleotides were purified by polyacrylamide gel electrophoresis followed by DEAE cellulose chromatography. The radioisotope [ $\alpha$ -32p] UTP (800 Ci/mmol) was from Amersham. Enzymes for digesting and ligating DNA were purchased from New England Biolabs, and used according to the supplier's

recommendations. Preparations containing the large fragment of DNA polymerase 1 (Klenow) were also purchased from New England Biolabs. RNasin and T7 RNA polymerase from Promega Biotec were purchased through Bio/Can Scientific Inc. Reverse transcriptase and RNase H were obtained from Pharmacia. The supplier for proteinase K was Boehringer Mannheim Canada. *E. coli* strain HB101 (ATCC 33694) was used for all transformations. The plasmid pUC19 (Norrander et al., 1983) was purchased from Bethesda Research Laboratories.

#### Isolation of DNA and sequencing

*E. coli* transformants were grown on YT medium (Miller, 1972) containing 50 µg/ml ampicillin. Plasmid DNA was purified by a rapid boiling method (Holmes and Quigley, 1981). DNA fragments and vectors used for all constructions were separated by electrophoresis on low melting point agarose, and purified from the molten agarose by phenol extraction and ethanol precipitation (Maniatis et al., 1982). Plasmid DNA was sequenced using a modification (Hattori et al., 1985) of the dideoxy method (Sanger et al., 1977). Reactions were run using the -20 universal primer (New England Biolabs).

**TCA precipitation**

Aliquots (5 $\mu$ l) of amplification reactions were quenched in 20 $\mu$ l 10 mM EDTA and placed on ice until all time point samples had been collected. The quenched samples were then applied to glass filter discs, and immediately dropped into ice-cold 5% trichloroacetic acid ("TCA") - 1% sodium pyrophosphate for 10 min with occasional mixing. Two 5 min washes with ice-cold 5% TCA were followed by two additional washes with 95% ethanol and lyophilization to dryness. Radioactivity was determined in a liquid scintillation counter.

**Polyacrylamide gel electrophoresis**

Samples (1 to 6  $\mu$ l) were mixed with 4-5  $\mu$ l formamide dye (90% deionized formamide, 10 mM TrisHCl (pH 8.0), 1 mM EDTA, xylene cyanol and bromophenol blue), and applied to a pre-run 12-cm- long 7% denaturing polyacrylamide gel. Gels were run at 350 volts until the bromophenol blue dye had reached the bottom. In some cases the gels were fixed and dried prior to autoradiography. Fixing involved a 15 min wash in 10% methanol- 7% acetic acid. The profiles of

the RNA products separated by this procedure were visualized by autoradiography at room temperature.

Example 1: Design and synthesis of oligonucleotides for a gag test system

A synthetic DNA sequence (Figure 2A) was designed to include an EcoRI site, a T7 phage promoter, a sequence required for initiation of transcription by T7 RNA polymerase and a 19 bp hybridization region (hybridization region 1). The 47 b strand oligonucleotide (T7H1.GAG) involved in the cloning of these elements also serves as primer.

Hybridization region 2 lies 53 bp away from hybridization region 1 and is 20 bp in length. The primer made to this region (H2.GAG) is a 20 b oligonucleotide duplicate of the sense strand and is not used for cloning. The sequence spanning and including the hybridization regions is a 92 bp segment of the gag portion of the HTLV-III genome. This particular gene segment was chosen because the primers were predicted to hybridize efficiently and because the distance between the two hybridization regions was relatively short. In addition, a XbaI site was placed at the end of the sequence for cloning ease. The gag

test sequence also contains SphI and PstI sites which can aid in the screening of recombinants.

A total of four oligonucleotides were used in the cloning of this fragment. N1.GAG, used in the construction of both the gag test and the gag2 test sequence, completes the antisense strand and is used only in the cloning process. Similarly, T74.PRO is the sense strand component of the T7 promoter. N2.GAG however, was used in the construction of both test fragments, and has also been used as an intermediate (second template) in two steps of the amplification cycle. The entire cloned gag test fragment can also represent an intermediate of the amplification cycle (third template). Once cloned into an appropriate vector the gag test DNA could be transcribed by T7 RNA polymerase to produce a RNA fragment (first template) useful as an amplification intermediate involved in three of the steps. In addition, T7H1.GAG and H2.GAG serve as primers in the test system.

The gag2 test synthetic DNA fragment (Figure 2B) does not contain the T7 promoter, but the remainder of the sequence is identical to the gag test sequence and therefore, both N1.GAG and N2.GAG were involved in its

construction. The oligonucleotide required to complete the antisense strand is called H1.GAG. Once cloned, the gag2 test fragment can be used as a template for testing amplification, using a DNA restriction fragment as template nucleic acid.

Example 2: Construction of the gag test plasmids

The oligonucleotides T74.PRO and N1.GAG (2  $\mu$ g each), were phosphorylated separately in 20  $\mu$ l reactions containing 70 mM TrisHCl (pH 7.6), 10 mM MgCl<sub>2</sub>, 5 mM DTT, 0.5 mM ATP and 5 units T4 polynucleotide kinase, at 37 °C for 30 min. Phosphorylated T74.PRO and N1.GAG (10  $\mu$ l of each) were mixed with 1  $\mu$ g each of unphosphorylated T7H1.GAG and N2.GAG, and 3  $\mu$ l 100 mM TrisHCl (pH 7.8) - 500 mM NaCl, in a final volume of 29  $\mu$ l for the gag test assembly. The gag2 test mixture contained 10  $\mu$ l phosphorylated N1.GAG, 1  $\mu$ g each of unphosphorylated H1.GAG and N2.GAG, and 1.8  $\mu$ l 100 mM TrisHCl (pH 7.8) - 500 mM NaCl, in a final volume of 18  $\mu$ l. The oligonucleotide mixtures were hybridized separately by placing them at 90 °C for 10 min followed by slow cooling to room temperature for 10 to 16 h 60  $\mu$ l reactions containing 50 mM TrisHCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 20 mM DTT, 1 mM

ATP and 50  $\mu$ g/ml BSA were used to ligate the hybridized oligonucleotides together. 400 units T4 DNA ligase was added to the gag test reaction and it was incubated at 15 °C for 2 h while the gag2 test reaction was incubated for 14 to 16 h with 200 units T4 DNA ligase.

The isolated and purified synthetic DNA segments were mixed with plasmid pUC19 which had been linearized by digestion at restriction enzyme sites within the polylinker region. T4 DNA ligase was used to ligate the gag test sequence into the EcoRI- XbaI fragment of pUC19, while the gag2 test sequence was ligated to the SmaI-XbaI fragment. Plasmid DNA from transformants obtained after these reactions were used to transform E. coli were screened by restriction analysis, and the final plasmids (pGAG.TEST and pGAG2.TEST) were determined to be correct by sequence analysis.

Example 3: Effect of primer concentration on RNA amplification

The reaction mixtures (25  $\mu$ l) which were used to amplify RNA transcribed from the gag test

oligonucleotides contained 50 mM TrisHCl (pH 8.45), 6 mM MgCl<sub>2</sub>, 40 mM KCl, 10 mM dithiothreitol, 0.5 mM NTP (ATP, CTP, GTP, UTP), 1 mM dNTP (dATP, dCTP, dGTP) dTTP), 20 units RNasin, 10 units T7 RNA polymerase, 10 units reverse transcriptase, 0.4 units RNase H, and 10  $\mu$ Cl [ $\alpha$ -32p] UTP. Two of the reactions contained 0.5 ng (0.015 pmoles) N2.GAG while the other two reactions contained no template. The primers T7H1.GAG and H2.GAG were each added at final concentrations of 3.4  $\mu$ M or 0.34  $\mu$ M to reactions containing either N2.GAG or no template. The reactions were incubated at 42 °C for 2 h. Total synthesis of RNA was monitored by determining the incorporation of TCA insoluble cpm at 30 min intervals. The effect of the primer concentration on template- dependent RNA synthesis is shown in Table 1. Aliquots of each reaction, containing equal amounts of synthesized RNA, were analyzed by PAGE and autoradiography (Figure 3, lanes 1-4 numbered the same as the reactions).

Table 1: RNA amplification from N2.GAG after 2 h.

<u>Reaction</u>	<u>Concentration of each primer (<math>\mu</math>M)</u>	<u>Template (ng)</u>	<u>RNA Synthesized (<math>\mu</math>g)</u>
1	3.4	0.5	2.8
2	3.4	-	2.1
3	0.34	0.5	1.8
4	0.34	-	0.7

It was found that while reaction 1 resulted in the greatest incorporation of isotope, the no template control, reaction 2, was also high (73% of reaction 1) and produced a very similar electrophoretic profile. It would therefore appear that in the presence of high primer concentrations, a RNA transcript of identical size to that expected in amplification is produced in the absence of any template. Results using samples with a 10-fold decrease in primer concentrations were dramatically different. The amount of RNA produced in reaction 3 was 2.6 times that of reaction 4, but while virtually all of the transcript was found in a single band of the expected size in reaction 3, no fragments greater than 60 to 70 b were found in reaction 4. Primer concentration therefore plays a significant role in the accuracy and efficiency of RNA amplification.

A control RNA transcript used to represent the size of fragment expected to be generated by the amplification system (lane 0 of Figure 3) was prepared by transcription from the test plasmid. pGAG.TEST was linearized by digestion with XbaI, proteinase K treated (Maniatis et al., 1982), phenol extracted and ethanol precipitated. T7 RNA polymerase was then used

according to the supplier's recommendations to transcribe 0.5  $\mu$ g of the resulting fragment in a 25  $\mu$ l reaction mixture containing 10  $\mu$ Ci [ $\alpha$ -32p] UTP.

Example 4: Effect of template concentration on RNA amplification.

The standard 50  $\mu$ l reaction mixture used to amplify RNA transcribed from the gag test oligonucleotides contained 0.34  $\mu$ M T7H1.GAG, 0.34  $\mu$ M H2.GAG, 50 mM TrisHCl (pH 8.45), 6 mM MgCl<sub>2</sub>, 40 mM KCl, 10 mM DTT, 0.5 mM NTP, 1 mM dNTP, 40 units RNasin, 20 units T7 RNA polymerase, 20 units reverse transcriptase, 0.8 units RNase H and 10 - 20  $\mu$ Ci [ $\alpha$ -32p] UTP. The reactions contained amounts of template (N2.GAG) varying from 1 ng to 1 fg. One reaction contained no template. The reactions were incubated at 42 °C for 3 h, during which total synthesis of RNA was monitored by determining the incorporation of TCA insoluble cpm at 30 min intervals. As indicated in Table 2, total RNA synthesis was higher than the no template control for all template concentrations tested. Although the total synthesis of RNA generally decreased with decreasing template concentration, this decrease in synthesis was not

quantitative. Thus, the degree of amplification of RNA per starting template generally increased with decreasing template concentration. Amplification of  $8 \times 10^8$  fold was achieved by synthesis of 0.8  $\mu$ g RNA from 1 fg of N2.GAG template. One fg of the 102-b N2.GAG oligonucleotide represents approximately  $2 \times 10^4$  molecules.

Table 2: RNA amplification from N2.GAG after 3 h.

<u>Reaction</u>	<u>Template</u>	RNA Synthesized ( $\mu$ g)	Fold amplification
1	1 ng	3.5	$3.5 \times 10^3$
2	100 pg	4.4	$4.4 \times 10^4$
3	10 pg	4.1	$4.1 \times 10^5$
4	1 pg	3.0	$3.0 \times 10^6$
5	100 fg	2.7	$2.7 \times 10^7$
6	10 fg	1.9	$1.9 \times 10^8$
7	1 fg	0.78	$7.8 \times 10^8$
8	-	0.046	-

The RNA which was synthesized after a reaction time of 3 h was analyzed by PAGE, for each template concentration (Figure 4 lanes 1-8, numbered the same as the reactions). A major band representing a RNA of about 100 b was present in all reactions except the ones containing 1 fg template and no template. The reaction containing 1 fg template did not have much of this 100 b product at 3 h but the overall RNA

synthesis was higher than and qualitatively different from the no template reaction.

Example 5: Hybridization analysis of RNA products.

Amplification reactions containing amounts of N2.GAG template varying from 1 pg to 0.1 fg were performed following the teaching of example 4, except the radiolabeled UTP was omitted. The reactions were incubated at 42 °C for 3 h. Aliquots were removed from each reaction at 30 min intervals and applied to a nylon membrane (Amersham). The nucleic acids that were contained in these reaction aliquots were fixed by exposure to ultraviolet light. The membrane was prehybridized at 50 °C for 1 h in prehybridization buffer consisting of a final concentration of 50% v/v formamide, 5 x SSC and 5 x Denhardt's solution (Maniatis *et al.*, 1982; Southern *et al.*, 1975) at a volume equivalent to 5 mls of solution per 100 cm<sup>2</sup> and hybridized with a radiolabeled probe with a specific activity of 10<sup>6</sup> cpm/ml of hybridization solution. Hybridization was performed at 50 °C for 16 h in 50% formamide, 5 X SSC and 5 X Denhardt's solution (Maniatis *et al.*, 1982; Southern *et al.*, 1975). The radiolabeled probe was the synthetic

oligonucleotide 5' GATCTGGGATAGAGTACATCCA 3' which had been labeled at the 5' end using T4 polynucleotide kinase and ( $\alpha$ -32p) ATP. After the membrane was washed at 50 °C in a series of 2, 3 min. washes consisting of 2 X SSC, 0.1% v/v SDS and 0.2 X SSC, 0.1% v/v SDS (Southern *et al.*, 1975; Maniatis *et al.*, 1982; Szostak *et al.*, 1979).

Figure 5 shows the results of the hybridization analysis performed on the amplification reactions, containing various amounts of N2.GAG template, which were sampled at different times of incubation.

Each column of Figure 5 represents a different time point (1, 30 min; 2, 60 min; 3, 90 min; 4, 120 min; 5, 150 min; 6, 180 min) and each row represents a different amount of added N2.GAG template (1, 1pg; 2, 100 fg; 3, 10 fg; 4, 1 fg; 5, 0.1 fg; 6, no template). Amplification of nucleic acids which hybridized to the labeled probe were observed for rows 1-3 (1 pg - 10 fg), however the hybridization to specific nucleic acids in rows 4 - 5 (1 fg, 0.1 fg) was not higher than row 6 (no template). The apparent non-specific binding of labeled probe in row 6 seems to be related to DNA or RNA synthesis since the hybridization signal increases with increasing time.

Example 6: Use of DNA restriction fragment as template.

The plasmid pGAG2.TEST was digested with MspI and proteinase K, purified by phenol extraction and ethanol precipitation, and denatured by boiling for 5 min. Amplification reactions were performed and analyzed following the teaching of Example 4, except the MspI digested pGAG2.TEST was used as a template instead of the N2.GAG oligonucleotide. The amounts of plasmid added to each reaction varied from 55 ng to 5.5 pg, and no template. To simulate additional DNA which would be present in an actual sample, alternate reactions contained 1 ng of calf thymus DNA which had been similarly digested, purified and denatured. After a 3 h incubation at 42 °C, the synthesis of RNA was determined by TCA precipitation and PAGE analysis. As indicated in Table 3, total RNA synthesis was higher than the no template controls for all template concentrations tested. The degree of amplification was calculated based on RNA synthesis from the actual template which was 1.8% of the total plasmid DNA.

The total RNA synthesis (degree of amplification) from a particular initial level template concentration

was consistently lower for the restriction fragment (Table 3) as compared to that for the synthetic oligonucleotide template (Table 2). This could be due to competition with the complementary strand of the restriction fragment template under the conditions used.

Table 3: RNA amplification from MspI-digested pGAG2.TEST

<u>Reaction</u>	<u>Template*</u>	<u>RNA Synthesized**</u>	<u>Fold amplification**</u>
1	55.0 ng [1 ng]	3.65	$3.7 \times 10^3$
2		(4.05)	$(4.1 \times 10^3)$
3	5.5 ng [100 pg]	3.54	$3.5 \times 10^4$
4		(3.16)	$(3.2 \times 10^4)$
5	550.0 pg [10 pg]	2.29	$2.3 \times 10^5$
6		(2.79)	$(2.8 \times 10^5)$
7	55.0 pg [1 pg]	2.62	$2.6 \times 10^6$
8		(0.67)	$(0.7 \times 10^6)$
9	5.5 pg [100 fg]	1.37	$1.4 \times 10^7$
10		(2.26)	$(2.3 \times 10^7)$
11	-	1.25	-
12		(0.08)	

\* Numbers in brackets indicate equivalent amounts of N2.GAG.

\*\* Numbers in parentheses indicate RNA synthesis in presence of 1  $\mu$ g MspI-digested calf thymus DNA.

The RNA which was synthesized after a reaction time of 3 h was analyzed by PAGE (Figure 6, lanes 1-6, 11 and 12. numbered the same as the reactions). The major band representing a RNA of about 100 b was present in reactions (lanes) 1-6 but absent in the no template reactions (lanes 11 and 12). The RNA in lane 0 was a standard, which was prepared following the teaching of Example 3. There was no apparent qualitative difference in the synthesized RNA either with (lanes 2, 4 and 6) or without (lanes 1, 3, and 5) the addition of 1  $\mu$ g of MspI-degested calf thymus DNA.

Example 7: Indirect Amplification

MATERIALS AND METHODS

(a) Bacterial Strains and Plasmids

A pGEM.4-gag plasmid and an M13-Mp18-gag plasmid each containing a 1422 base pair XbaI-EcoRI restriction fragment from HIV 1 (strain BH10) were constructed from a SacI-BgIII subclone obtained as a gift from Dr. P. Gill, President of the Canadian Red Cross Society. This restriction fragment contains the majority of the HIV1 gag gene (Ratner,

L.). *E. coli* strain HB101 was transformed with the pGEM4-gag plasmid and *E. coli* strain TGI was transformed with the M13-gag plasmid. Plasmid DNA was prepared by methods described in Maniatis et al. (Maniatis, I., Fritsch, E.F. and Sambrook, J.).

(b) Synthesis of RNA Template

To obtain gag-RNA template, the pGEM.gag plasmid was linearized with XbaI, extracted with phenol-chloroform and precipitated in ethanol. Purified DNA was transcribed using SP6 RNA polymerase (Promega) according to the method of Melton (Melton, D.A. et al.). 5 units of RNase-free DNaseI (Promega) was added and the mixture incubated at 37 °C for 30 minutes. The RNA product was extracted with phenol-chloroform and precipitated with ethanol. The yield of RNA was determined spectrophotometrically.

(c) Synthesis of Oligonucleotides and Preparation of End-Labelled Probe

Oligonucleotides were synthesized on an Applied Biosystems Model 380-A DNA synthesizer. DNA oligomers were purified by polyacrylamide gel electrophoresis

and DE 52 column chromatography. Synthesis yields were determined spectrophotometrically using a conversion factor of 40  $\mu$ g/A260 unit.

The first primer was a 20-mer; the second primer was 45 bases in length (the T7 promoter sequence is underlined) and the probe was a 53-mer. These sequences were as follows:

FIRST PRIMER: 5' ACA TCA AGC CAT GCA AA 3'

SECOND PRIMER: 5' AAT TCT AAT ACG ACT CAC TAA AGG GAG  
TAG TTC CTG CTA TGT CAC 3'

probe: 5' TGT TAA AAG AGA CCA TCA ATG AGC AAG CTG  
CAG AAT GGG ATA GAG TAC ATC CA 3'

The 53-base oligonucleotide probe was complimentary to the amplification product sequence and was labelled by Polynucleotide Kinase (BRL) in the presence of X-32P ATP (Amersham).

Amplification Reactions

RNA targets induced the indirect amplification reaction in the milieu containing 50 mM Tris-HCl, pH 8.3. 6 mM MgCl<sub>2</sub>, 40 mM KCl, 10 mM DTT, 1 mM deoxyribonucleotides (Pharmacia) 0.5 mM ribonucleotides (Pharmacia) 40 units of AMV-reverse transcriptase (Seikagaku). 0.4 units RNase H (Pharmacia), 20 units T7 RNA polymerase (Pharmacia) and 0.5 units per  $\mu$ l RNA-Guard<sup>TM</sup> (Pharmacia). The concentration of each primer was 0.35  $\mu$ M and the final reaction volume was 25  $\mu$ l. Standard reactions were run at 42 °C for 3 hours and were terminated with the addition of EDTA to a final concentration of 5 mM.

DNA targets were amplification-induced as for RNA with the following modifications. Double-stranded DNA was mixed with buffer, nucleotides and primers, heated to 95 °C for 3 minutes, and then placed on ice. T7-DNA polymerase (Sequenase<sup>TM</sup> from United States Biochemical) was added to a final concentration of 0.5 units per  $\mu$ l and incubated for 15 minutes at 37 °C. The mixture was heated to 95 °C for 3 minutes and then cooled, whereupon DTT, reverse transcriptase, RNase H, T7 RNA polymerase and RNA-Guard<sup>TM</sup> were added, and the reaction run at 42 °C for 3 hours.

Hybridization Analysis of Amplification Products

A 5  $\mu$ l sample of each reaction was glyoxylated by the method recommended in Maniatis et al. (Maniatis, I., Fritsch, E.F. and Sambrook, J.), mixed with 200  $\mu$ l of 6 X SSC and bound to a nylon membrane (Amersham) using a slot-blot apparatus (Bio-Rad<sup>TM</sup>). The nucleic acid was fixed to the membrane by an 8-minute treatment of the filter in 0.1 N NaOH. The filter was hybridized with 32 P-end-labelled oligonucleotide in 50% formamide, 3 X SSC and 5 X Denhardt's solution (Denhardt, D.T.) at 42 °C for 2 hours. Hybridized filters were washed for 10 minutes at room temperature in 2 X SSC and 0.5% SDS followed by 1 hour in 0.1% SSC, 0.5% SDS at 50 °C. Autoradiography was performed using Kodak XAR-5 film.

Sequence Analysis of Amplification Products

Amplification reactions were treated with 2 units of RNase-free DNaseI for 30 minutes at 37 °C, followed by phenolchloroform extraction and ethanol precipitation. Purified RNA was hybridized with a 32P end-labelled primer and sequenced using reverse transcriptase in the presence of dideoxynucleotides (Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L. and Pace, N.R.).

RESULTS

The indirect amplification process is shown in Figure 7. With a natural RNA target (mRNA, nRNA, tRNA, etc.) the indirect amplification reaction proceeds with the annealing of the second primer which contains a T7-RNA polymerase promoter sequence at its 5' end (19,20). Reverse transcriptase present in the reaction mix generates a complementary DNA strand from the 3' end of the second primer. The RNA strand -- the natural RNA target -- of the RNA/DNA duplex is degraded by RNase H present in the mix. The first primer anneals to the resultant single strand of cDNA and second-stranded synthesis occurs, rendering the promoter sequence double-stranded. From this functional promoter, the T7-RNA polymerase present in the mix generates multiple copies of RNA, antisense to the original target, which serve as new templates for cDNA synthesis in the cyclic phase of the indirect amplification reaction. This cyclic phase is characterized by the primers annealing in the reverse order to the non-cyclic phase: that is, the first primer anneals first, initiating the reverse transcriptase and RNase H reactions, followed by the second primer priming double-stranded DNA synthesis, again creating a functionally active T7 promoter.

Figure 7 also illustrates where DNA intermediates occur in the indirect amplification process and therefore how DNA targets themselves can be the subject of exponential amplification via the indirect amplification reaction. Double-stranded target DNA is cut with a restriction enzyme and denatured to create molecules with defined ends, whereupon the second primer anneals and initiates cDNA synthesis, thus creating a functionally active T7 promoter which generates RNA competent to induce the cyclic portion of the indirect amplification process. If unrestricted double-stranded DNA is used as a target, then, as Figure 7 illustrates, both the first primer and the second primer are required to generate a functionally active T7 promoter. Nevertheless, the same indirect amplification process generates the amplified product, whether RNA or DNA is the target.

In order to demonstrate the feasibility of this indirect amplification system, both RNA and DNA templates were tested. The pGEM-gag plasmid served as the source of double-stranded DNA template. To obtain RNA template, the pGEM-gag plasmid was linearized using *Xba*I, which cuts at the end of the inserted DNA. The linearized plasmid was transcribed by SP6 RNA polymerase to yield an RNA product 1422 bases in

length. Primer sites were selected such that the amplified product from either the DNA or RNA target was 140 bases in length.

The kinetics of amplification was determined for various amounts of RNA template under standard reaction conditions (see Materials and Methods). Table 4 shows that the indirect amplification reaction generates product from either RNA or DNA targets in an exponential fashion.

TABLE 4  
PRODUCT GENERATED BY INDIRECT AMPLIFICATION

<u>INITIAL RNA TEMPLATE+</u>		<u>RNA PRODUCT</u>		<u>AMPLIFICATION</u>
MASS	#MOL.	MASS	#MOL.	
1.0 ng	$1.2 \times 10^9$	4.2 $\mu$ g	$5.0 \times 10^{13}$	$4.2 \times 10^4$
1.0 pg	$1.2 \times 10^6$	2.75 $\mu$ g	$3.3 \times 10^{13}$	$2.75 \times 10^7$
1.0 fg	$1.2 \times 10^3$	0.8 $\mu$ g	$9.6 \times 10^{12}$	$8.0 \times 10^8$

<u>INITIAL DNA TEMPLATE+</u>		<u>RNA PRODUCT</u>		<u>AMPLIFICATION</u>
MASS	#MOL.	MASS	#MOL.	
1.0 ng	$1.2 \times 10^9$	4.0 $\mu$ g	$4.7 \times 10^{13}$	$4.0 \times 10^3$
1.0 pg	$1.2 \times 10^6$	2.6 $\mu$ g	$3.1 \times 10^{13}$	$2.6 \times 10^7$
1.0 fg	$1.2 \times 10^3$	0.72 $\mu$ g	$8.6 \times 10^{12}$	$7.2 \times 10^8$

+ RNA and DNA templates are 1422 bases in length.

\* Amplification product is 140 bases in length.

The specificity of the indirect amplification reaction was also examined. One nanogram and 1 picogram of RNA or DNA were targets in the indirect amplification reaction in the presence or absence of 1  $\mu$ g of heterologous RNA or DNA under standard conditions. Following agarose gel electrophoresis, the reaction products were examined by ethidium bromide staining. Only a single amplification product of approximately 140 bases in length was obtained in any of these reactions, thus indicating that the primer sequences selected were specific for the correct segment of the gag gene. The presence of heterologous total RNA isolated from HL60 cells had no effect on the specificity of the indirect amplification reaction. Densitometer scans of the 140 base product indicated that a 1000-fold reduction in template (1pg  $\rightarrow$  1fg) resulted in only a 50% reduction in product yield. The presence of HL60 RNA reduced the yield by approximately 5-fold. Similar experiments with gag-RNA amplified in the presence of 1  $\mu$ g of calf thymus DNA showed less than a 2-fold reduction in product compared to gag-RNA alone. Similar quantitative results were obtained when the reaction products were analyzed by hybridization. Even with a five-fold decrease in product due to the

HL60 RNA, the 1fg sample was readily datactable by hybridization.

The sensitivity of the indirect amplification reaction was determined for both DNA and RNA targets. Stock solutions of each target were diluted serially in sterile water containing 20  $\mu$ g/ml RNase-free E. coli tRNA. Aliquots of these dilutions were subjected to standard indirect amplification conditions for 3 hours at 42 °C. The 32P-end-labelled oligonucleotide probe was used to detect amplification products bound to nylon membranes. Both DNA and RNA targets were detectable starting with as few as 10 initial molecules.

The nucleotide sequence of the RNA product from standard indirect amplification reactions was examined. One picogram of either DNA or RNA target was subjected to indirect amplification conditions for 3 hours at 42 °C and the products were sequenced by extension from 32P-labelled P2 primer in the presence of dideoxynucleotides. The products of the sequencing reactions were separated in an 8% acrylamide gel. Analysis of the sequence obtained indicated that not only was the product of correct length (140 bases),

but also the sequence obtained was identical to the correct segment of the gag gene.

This indirect amplification process amplifies a small number of nucleic acid molecules greater than  $10^8$ , in an indirect manner, in three hours in the presence of greater than a million-fold excess of heterologous nucleic acid.

The following are the features of this indirect nucleic acid amplification process:

First, the entire indirect amplification reaction is homogenous, that is, no additions are made to the reaction once it is begun;

Second, the indirect amplification reaction takes place at a single temperature;

Third, the principal product of the indirect amplification reaction is RNA complementary to the original target. This single-stranded product is readily detected without further manipulation;

Fourth, while the majority of the reaction product is RNA, there are formed during the indirect

amplification reactions double-stranded DNA molecules which are essential to the process. Sufficient DNA is generated to allow cloning directly from the reaction;

Fifth, in the vectorial phase of the indirect amplification reaction, the second primer is used to initiate cDNA synthesis, whereas in the cyclic phase of the indirect amplification reaction, cDNA synthesis is initiated with the first primer (see Figure 7);

Sixth, the simple indirect amplification format is accomplished by the simultaneous enzymatic synthesis and degradation of RNA in such a way that transcriptionally functional DNA accumulates.

While the overall amplification of the indirect amplification process is difficult to quantify mathematically, it most likely involves a third-order polynomial expansion.

Although the indirect amplification reaction reported here was run for a standard three-hour period, this time could be reduced (to as little as 30 minutes) for diagnostic purposes. The time of reaction depends upon the estimated amount of target

nucleic acid present, the detection system used, and the amount of detectable product which gives significant signal to background ratios.

Although preferred embodiments of the invention have been described in detail, it will be understood by those skilled in the art that variations may be made thereto without departing from either the spirit of the invention or the scope of the appended claims.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A process for amplifying a specific nucleic acid sequence comprising:

synthesizing single-stranded RNA, single-stranded DNA and double stranded DNA in which the single stranded RNA is a first template for a first primer, the single stranded DNA is a second template for a second primer, the double stranded DNA is a third template for synthesis of a plurality of copies of the first template, where a sequence of the first or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence, a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence, and a 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

2. A process as set forth in claim 1 in which the first primer selectively hybridizes to the first template and promotes the synthesis of DNA covalently attached to the first primer and complementary to the first

template; and the second primer; having a sequence for a promoter and a sequence for a transcription initiation site for a RNA polymerase, selectively hybridizes to the second template thereby permitting the synthesis of DNA covalently attached to the second primer and complementary to the second template.

3. A process for amplifying a specific nucleic acid sequence comprising:

- (a) hybridizing a first primer of DNA to a first template, the first primer having a sequence sufficiently complementary to a RNA sequence of the first template;
- (b) synthesizing a first DNA sequence covalently attached to the first primer and complementary to the RNA sequence of the first template, wherein the first DNA sequence and the first primer comprise a second template;
- (c) separating the first template from the second template to allow hybridization of a second primer;
- (d) hybridizing the second primer of DNA to the second template, the second primer having a sequence sufficiently complementary to a DNA sequence of the second template, the second primer having a sequence of a promoter and a sequence of a transcription initiation site for a RNA polymerase;
- (e) synthesizing a second DNA sequence covalently attached to the second primer and complementary

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to the DNA sequence of the second template and synthesizing a third DNA sequence covalently attached to the second template and complementary to the DNA sequence of the second primer wherein the second and third DNA sequences, the second primer and the second template comprise a third template;

(f) synthesizing a plurality of copies of the RNA sequence of the first template from the third template;

where a sequence of the first or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence, a sequence of the

first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence, and a 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

4. A process as set forth in claim 3 in which the second primer has a sequence at its 3' end sufficiently complementary to the DNA sequence of the second template and has at its 5' end a DNA sequence of a promoter and a sequence of a transcription initiation site for a RNA polymerase.
5. A process as set forth in claim 4 in which the third DNA sequence covalently attached to the second template is complementary to the DNA sequence at the 5' end of the second primer.
6. A process as set forth in claim 3 in which the first DNA sequence is synthesized by a RNA directed DNA polymerase.
7. A process as set forth in claim 3 in which the first template is separated from the second template by a ribonuclease specific for hybrids of RNA-DNA.

8. A process as set forth in claim 7 in which the ribonuclease is E. coli ribonuclease H.
9. A process as set forth in claim 7 in which the ribonuclease is calf thymus ribonuclease H.
10. A process as set forth in claim 3 in which the second and third DNA sequences are synthesized by a DNA directed DNA polymerase.
11. A process as set forth in claim 3 which the first template is synthesized from the third template by a DNA directed RNA polymerase.
12. A process as set forth in claim 3 in which the first primer has a sequence of a promoter and a sequence of a transcription initiation site.
13. A process as set forth in claim 3 in which the first primer has a sequence which hybridizes to a support means.
14. A process as set forth in claim 3 in which the second primer has from its 3' to its 5' end, a sufficiently complementary sequence, a sequence of a transcription initiation site, and a sequence of a promoter.

15. A process as set forth in claim 14 in which the sequence of the transcription initiation site and the promoter sequence bind a bacteriophage RNA polymerase.
16. A process as set forth in claim 14 in which the sequence of the transcription initiation site and the promoter sequence bind T7 RNA polymerase.
17. A process as set forth in claim 14 in which the sequence of the transcription initiation site and the sequence of the promoter is AATTCTAATACGACTCACTATAGGGAG.
18. A process as set forth in claim 11 in which the DNA directed RNA polymerase is T7 RNA polymerase.
19. A process as set forth in claim 11 in which the DNA directed RNA polymerase is a bacteriophage RNA polymerase.
20. A process as set forth in claim 11 in which the DNA directed RNA polymerase is a phage T3 polymerase.
21. A process as set forth in claim 11 in which the DNA directed RNA polymerase is phage  $\phi$ II polymerase.

22. A process as set forth in claim 11 in which the DNA directed RNA polymerase is *salmonella* phage *sp6* polymerase.
23. A process as set forth in claim 11 in which the DNA directed RNA polymerase is *Pseudomonas* phage *gh-1* polymerase.
24. A process as set forth in claim 6 in which the RNA directed DNA polymerase is a retroviral polymerase.
25. A process as set forth in claim 24 in which the retroviral polymerase is an avian myoblastosis viral polymerase.
26. A process as set forth in claim 24 in which the retroviral polymerase is a Maloney murine leukemia viral polymerase.
27. A process as set forth in claim 6, 10 or 11 in which the polymerase lacks exonuclease or endonuclease activity.
28. A process as set forth in claim 10 in which the DNA directed DNA polymerase is an avian myoblastosis viral polymerase.

29. A process for amplifying a specific nucleic acid sequence comprising:

combining a first primer, a second primer, ribonuclease H, RNA-directed DNA polymerase, DNA directed DNA polymerase, RNA polymerase, ribonucleoside triphosphates and deoxyribonucleoside triphosphates with a sample, where the first primer of DNA has a sequence sufficiently complementary to a first template of RNA; the second primer of DNA has a sequence sufficiently complementary to a second template of DNA, a sequence of a promoter, a sequence of a transcription initiation site which are recognized as substrate by the RNA polymerase;

where a sequence of the first or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence, a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence, and a 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

30. A process for amplifying a specific nucleic acid sequence comprising:

combining a first primer, a second primer, avian myoblastosis viral polymerase, E. coli ribonuclease H, bacteriophage T7 RNA polymerase, ribonucleoside triphosphates and deoxyribonucleoside triphosphates with a sample, where the first primer has a sequence sufficiently complementary to a first template of RNA; the second primer has a sequence sufficiently complementary to a second template of DNA, a sequence of a promoter and a sequence of a transcription initiation site which are recognized as substrate by T7 RNA polymerase;

where a sequence of the first or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence, a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence, and a 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

31. A process as set forth in claim 1 or 3 further comprising detecting the presence of the specific nucleic acid sequence by a comparison between an amount of amplification of a first sample suspected to contain the nucleic acid sequence and an amount of amplification of a second sample in which the nucleic acid sequence is not present.
32. A process as set forth in claims 29 or 30 further comprising detecting the presence of the specific nucleic acid sequence by a comparison between an amount of amplification of a first sample suspected to contain the nucleic acid sequence and an amount of amplification of a second sample in which the nucleic acid sequence is not present.
33. A process as set forth in claims 1 or 3 further comprising detecting the presence of the specific nucleic acid sequence with a probe which hybridizes to the specific nucleic acid sequence or a product of the specific nucleic acid sequence.
34. A process as set forth in claims 1 or 3 further comprising detecting the presence of the specific nucleic acid sequence using restriction endonucleases and electrophoretic separation.

35. A process as set forth in claims 31 or 32 further comprising detecting the presence of the specific nucleic acid sequence using restriction endonucleases and electrophoretic separation.
36. A process as set forth in claims 31 or 32 further comprising detecting the presence of the specific nucleic acid sequence with a probe which hybridizes to the specific nucleic acid sequence or a product of the specific nucleic acid sequence.
37. The specific nucleic acid sequence amplified by the process set forth in claim 1.
38. The specific nucleic acid sequence amplified by the process set forth in claim 3.
39. The specific nucleic acid sequence amplified by the process set forth in claim 29.
40. The specific nucleic acid sequence amplified by the process set forth in claim 30.
41. A kit for amplifying a specific nucleic acid sequence comprising:

- (a) first primer having a DNA sequence sufficiently complementary to a RNA sequence of a first template;
- (b) second primer having a DNA sequence complementary to a DNA sequence of a second template;
- (c) ribonuclease H;
- (d) RNA-directed DNA polymerase;
- (e) DNA-directed RNA polymerase;
- (f) DNA directed DNA polymerase;
- (g) ribonucleoside triphosphates; and
- (h) deoxyribonucleoside triphosphates;

where a sequence of the first or the second primer is sufficiently complementary to a sequence of the specific nucleic acid sequence, a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence, and a 3' end of the first primer is oriented towards

a 3' end of the second primer on complementary strands.

42. A kit for amplifying a specific nucleic acid sequence comprising:

- (a) first primer having a DNA sequence sufficiently complementary to a RNA sequence of a first template;
- (b) second primer having a DNA sequence sufficiently complementary to a DNA sequence of a second template;
- (c) bacteriophage T7 RNA polymerase;
- (d) *E. coli* ribonuclease H;
- (e) avian myoblastosis viral polymerase;
- (f) ribonucleoside triphosphates; and
- (g) deoxyribonucleoside triphosphates;

where a sequence of the first or the second primer is sufficiently complementary to a sequence of the

specific nucleic acid sequence, a sequence of the first or the second primer is sufficiently homologous to a sequence of the specific nucleic acid sequence, and a 3' end of the first primer is oriented towards a 3' end of the second primer on complementary strands.

43. A process for amplifying nucleic acid sequences, without serial addition of reagents or temperature recycling, comprising the steps of
  - (A) providing a single reaction medium containing a first oligonucleotide primer and a second oligonucleotide primer, at least one of which primers contains a sequence that is sequence of a functional promoter; an RNA-directed DNA polymerase; a DNA-directed DNA polymerase; a DNA-directed RNA polymerase; a ribonuclease that releases DNA of a RNA/DNA hybrid without attacking single- or double-stranded RNA or DNA; and ribonucleoside and deoxyribonucleoside triphosphates,
  - (B) adding to said reaction medium one or more of the following:

- (i) an RNA molecule;
- (ii) a single-stranded DNA molecule comprising:
  - (a) a 3'-end sequence that hybridizes to a region at the 3' end of said first oligonucleotide primer; or
  - (b) a 5'-end sequence that is a sequence of a functional promoter; or
  - (c) a 3'-end sequence that hybridizes to a region at the 3' end of said second oligonucleotide primer,
- (iii) a double-stranded DNA molecule comprising:
  - (i) at least one single stranded DNA described in B(ii); or
  - (ii) an amplifiable region and a functional promoter, the functional promoter being adjacent to said region and being oriented to control transcription of said region,

(C) at a relatively constant temperature, establishing conditions such that a cycle of events is initiated in said reaction medium, said cycle comprising at least one of the following sets of events:

FIRST SET OF EVENTS

- (i) said first oligonucleotide primer hybridizes to a region at the 3' end of an RNA molecule, and an RNA/DNA hybrid is formed by action of said RNA-directed DNA polymerase, said RNA/DNA hybrid comprising a first DNA segment, said first DNA segment being covalently attached to said first oligonucleotide primer and being complementary to at least a part of said RNA molecule;
- (ii) said ribonuclease acts on said RNA/DNA hybrid to digest at least some of said RNA molecule, releasing said first DNA segment from said RNA/DNA hybrid, and said first DNA segment hybridizes with said second oligonucleotide primer to form a duplex that is acted upon by said DNA-directed DNA polymerase to produce (a) a second DNA segment which is covalently

attached to said second oligonucleotide primer and which is complementary to at least a part of said first DNA segment and (b) a third DNA segment which is covalently attached to said first DNA segment and which is complementary to at least a part of said second primer; and

(iii) said RNA polymerase produces a plurality of RNA molecules that correspond to the RNA molecule of (C)(i);

and

SECOND SET OF EVENTS

(i') said second oligonucleotide primer hybridizes to a region at the 3' end of an RNA molecule, and an RNA/DNA hybrid is formed by action of said RNA-directed DNA polymerase, said RNA/DNA hybrid comprising a second DNA segment, said second DNA segment being covalently attached to said second oligonucleotide primer, and complementary to at least part of said RNA molecule;

(ii') said ribonuclease acts on said RNA/DNA hybrid to digest at least some of said RNA molecule, releasing said second DNA segment from said RNA/DNA hybrid, and said second DNA segment hybridizes with said first oligonucleotide primer, forming a duplex that is acted upon by said DNA-directed DNA polymerase to produce a first DNA segment which is covalently attached to said first oligonucleotide primer and which is complementary to at least a part of said second DNA segment; and

(iii') said RNA polymerase produces a plurality of RNA molecules that correspond to a molecule complementary to the RNA molecule of C(i')

and then

(D) maintaining said conditions until a predetermined concentration of at least one from the group consisting of; said RNA molecules of (C)(iii) or (C)(iii'), said first DNA segment, said second DNA segment, and said double-stranded DNA is detectable.

44. A process according to Claim 43, wherein step (B) comprises adding an RNA molecule antisense to the RNA molecule of (C)(i).
45. A process according to Claim 43, wherein step (B) comprises adding an RNA molecule antisense to the RNA molecule of (C)(i').
46. A process according to Claim 43, wherein step (B) comprises adding an RNA molecule to said reaction medium.
47. A process according to Claim 43, wherein step (B) comprises adding a single-stranded DNA molecule to said reaction medium.
48. A process according to Claim 43, wherein step (B) comprises adding a double-stranded DNA molecule to said reaction medium.
49. A process according to Claim 43, wherein step (C) comprises said first set of events.
50. A process according to Claim 43, wherein step (C) comprises said second set of events.

51. A process according to Claim 43, wherein step (D) comprises monitoring the concentration of RNA molecules of (C)(iii).
52. A process according to Claim 43, wherein step (D) comprises monitoring the concentration of RNA molecules of (C)(iii').
53. A process according to Claim 43, wherein step (D) comprises monitoring the concentration of first DNA segments.
54. A process according to Claim 43, wherein step (D) comprises monitoring the concentration of second DNA segments.
55. A process according to Claim 43, wherein step (D) comprises monitoring the concentration of double-stranded DNA.
56. A process according to Claim 43, wherein step (D) comprises maintaining said conditions for a period of time between 30 minutes and 4 hours.
57. A process according to Claim 43, wherein an RNA polymerase recognizes said sequence of a transcription initiation site and said

sequence of a functional promoter such that transcription is initiated.

58. A process according to Claim 43, wherein said DNA-directed DNA polymerase is DNA polymerase  $\alpha$  or S.
59. A process according to Claim 43, wherein said DNA-directed DNA polymerase is calf thymus DNA polymerase.
60. A process according to Claim 43, wherein said DNA-directed DNA polymerase is a DNA polymerase lacking exonuclease activity.
61. An RNA molecule or DNA molecule comprising a nucleic acid sequence amplified by the process according to Claim 43.
62. A process according to Claim 43, further comprising the steps of ligating said amplified nucleic acid sequence into a cloning vector and then cloning said nucleic acid sequence or expressing the product encoded by said nucleic acid sequence in an expression system.

63. A kit for amplifying nucleic acid molecules, comprising an assemblage of

- (a) a receptacle containing a solution of a first oligonucleotide primer,
- (b) a receptacle containing a solution of a second oligonucleotide primer,
- (c) a receptacle containing a solution of a ribonuclease that hydrolyses RNA of an RNA/DNA hybrid without attacking single- or double-stranded RNA or DNA,
- (d) a receptacle containing a solution of an RNA-directed DNA polymerase,
- (e) a receptacle containing a solution of a DNA-directed RNA polymerase,
- (f) a receptacle containing a solution of a DNA-directed DNA polymerase,
- (g) a receptacle containing a solution of ribonucleoside triphosphates and,
- (h) a receptacle containing a solution of deoxyribonucleoside triphosphates.

64. A process for amplifying nucleic acid sequences, at a relatively constant temperature comprising:

- (A) providing a single reaction medium containing a first oligonucleotide primer and a second oligonucleotide primer, which second primer comprises a sequence that is a single stranded sequence of a functional promoter; an RNA-directed DNA polymerase; a DNA-directed DNA polymerase; a DNA-directed RNA polymerase; a ribonuclease that releases DNA of a RNA/DNA hybrid without attacking ribonucleoside and deoxyribonucleoside triphosphates,
- (B) adding to said reaction medium
  - (i) an RNA molecule; or
  - (ii) a single-stranded DNA molecule comprising
    - a) a 3' end sequence that hybridizes to a region at the 3'-end of said first oligonucleotide primer; or
    - b) a 5'-end sequence that is a single-stranded sequence of a functional promoter; or
    - c) a 3'-end sequence that hybridizes to a region at the 3'-end of said second oligonucleotide primer; or

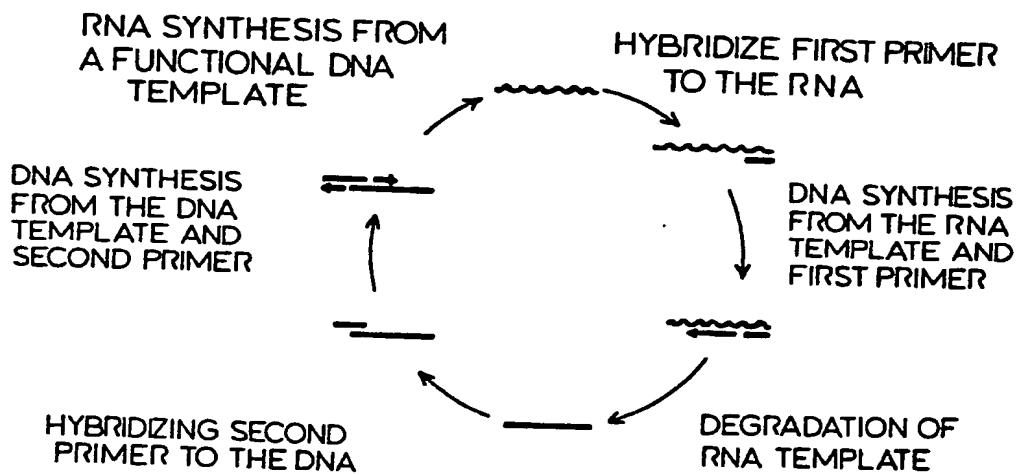
(iii) a double-stranded DNA molecule comprising:  
an amplifiable region and a functional  
promoter, the functional promoter being  
adjacent to said region and being oriented  
to control transcription of said region,

(C) establishing conditions such that a number of  
events is initiated in said reaction medium  
comprising at least the following steps:

- (i) said first oligonucleotide primer hybridizes to a region at the 3'-end of an RNA molecule, and an RNA/DNA hybrid is formed by action of said RNA-directed DNA polymerase, said RNA/DNA hybrid comprising a first DNA segment, said first DNA segment being covalently attached to said first oligonucleotide primer and being complementary to at least a part of said RNA molecule;
- (ii) said ribonuclease acts on said RNA/DNA hybrid to digest at least some of said RNA molecule, releasing said first DNA segment from said RNA/DNA hybrid, and said first DNA segment hybridizes with said second oligonucleotide primer at the 3'-end of said first DNA segment to form a duplex that is acted upon by said DNA-directed DNA polymerase to produce (a) a second DNA segment which is covalently attached to said second oligonucleotide primer and which is complementary to at least a part of said first DNA segment; and (b) a third DNA segment which is covalently attached to said first DNA segment and which is complementary to at least a part of said second primer, and
- (iii) said RNA polymerase produces a plurality of RNA molecules homologous to the RNA molecule of C(i).

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FIG.1.



A

FIG.2.

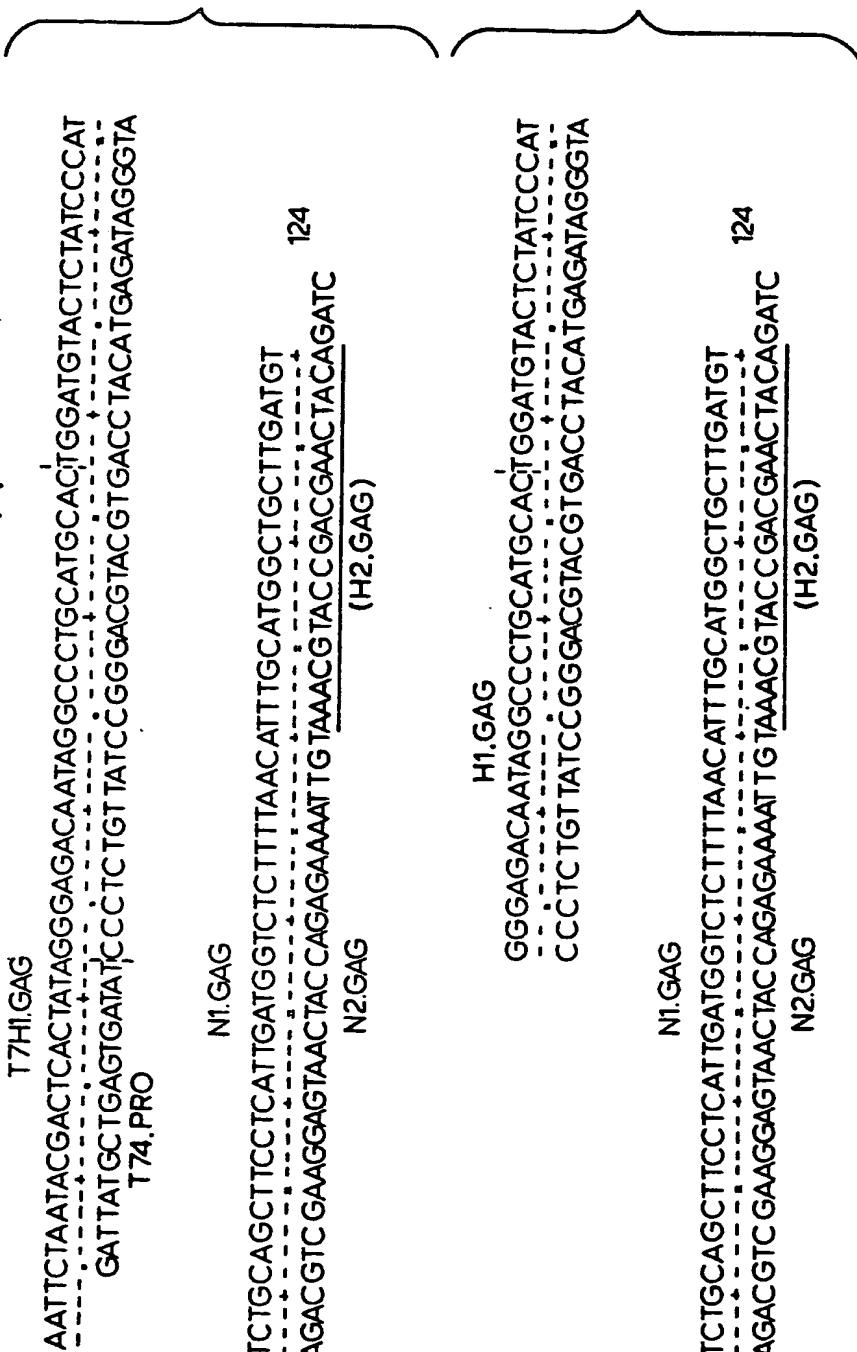


FIG.3.



FIG.4.



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FIG.5.

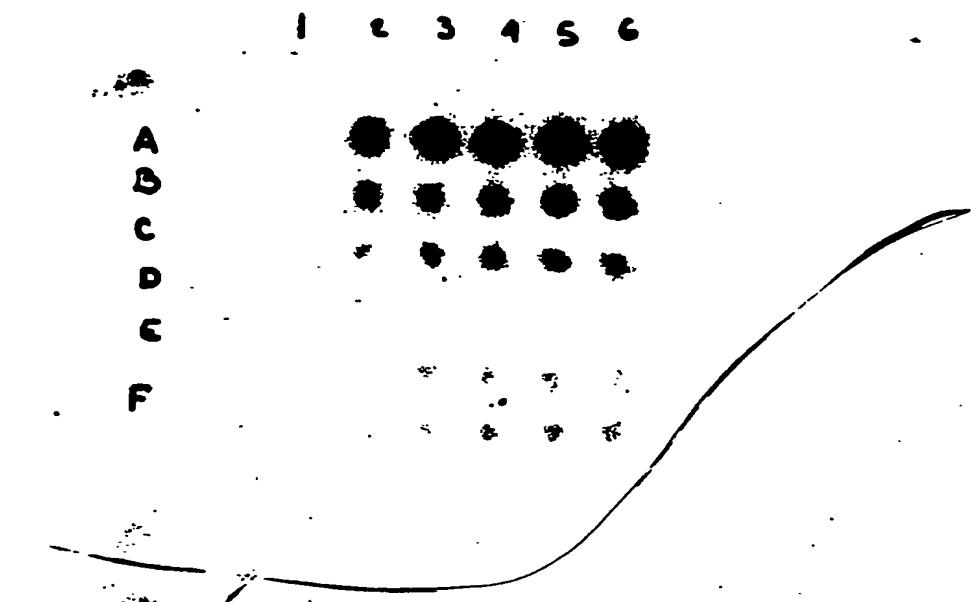
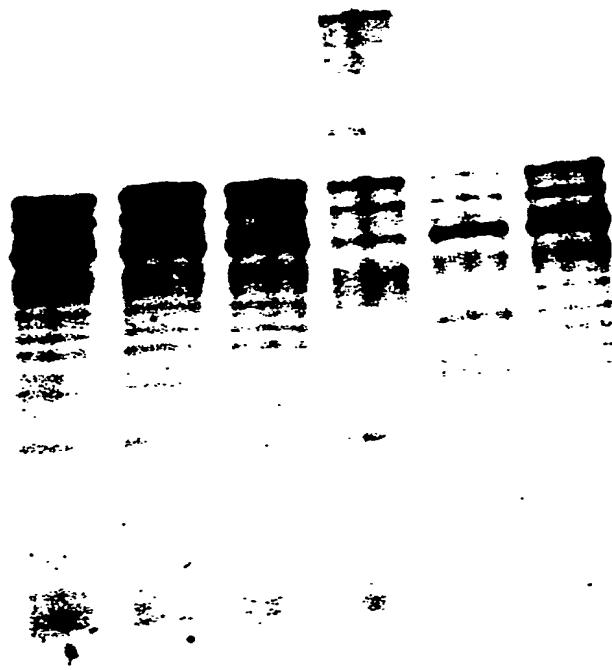


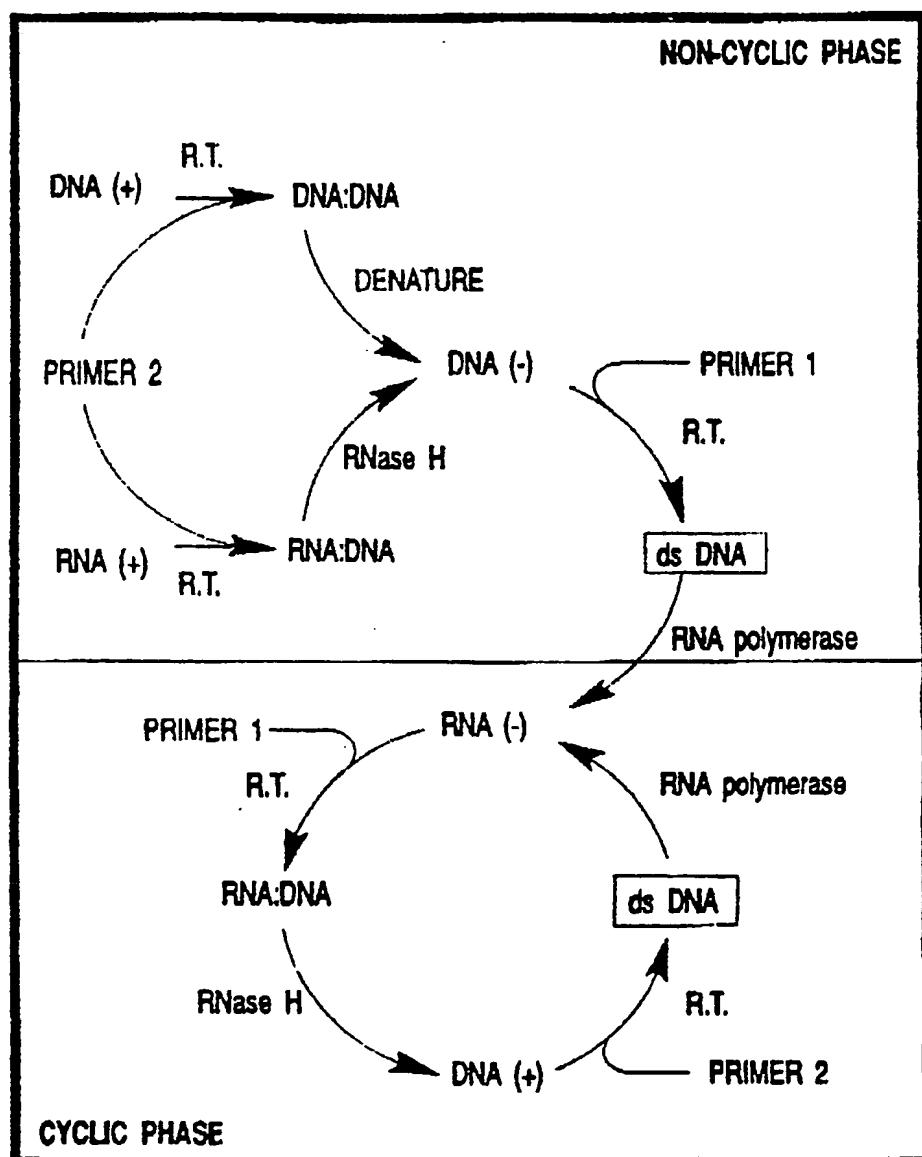
FIG. 6.



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Fig. 7



# INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 89/00981

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC5: C 12 Q 1/68 // C 07 H 21/04

## II. FIELDS SEARCHED

Minimum Documentation Searched ?

Classification System	Classification Symbols
IPC5	C 12 Q; C 12 N; C 07 H

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. 13
E	EP, A2, 0329822 (CANGENE CORPORATION) 30 August 1989, see the whole document	1-64
	---	
X	WO, A1, 88/10315 (SISKA DIAGNOSTICS, INC.) 29 December 1988, see especially the figures and claims	1-6, 10- 28, 31- 38
Y		7-9, 29- 30, 39- 64
	---	
Y	Dialog Information Services, File 351, World Patent Index 81-90, Dialog accession no. 88-096821/14, Yamashina I: "Gene, for supplying thrombin-like batroxobin enzyme - used for coding polypeptide contg. batroxobin amino acid sequence", JP 63049084, A, 880301, 8814 (Basic).	7-9, 29- 30, 39- 64
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\* Special categories of cited documents: <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

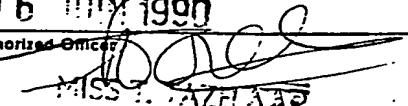
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search <b>10th April 1990</b>	Date of Mailing of this International Search Report <b>06 May 1990</b>
International Searching Authority <b>EUROPEAN PATENT OFFICE</b>	Signature of Authorized Officer  <b>MISS J. AZELEA</b>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Dialog Information Services, File 5, BIOSIS, BIOSIS number 69022711, Cathala G et al: "Isolation and characterization of 2 types of rnase H in krebs II ascites cells" J Biol Chem 254 (15), 1979, 7353-7359  --	7-9,29-30, 39-64
A	EP, A1, 0310229 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 5 April 1989 see the whole document  --	1-64
A	EP, A2, 0303155 (MERCK PATENT GESELLSCHAFT MIT BESCHRÄNKTER HAFTUNG) 15 February 1989 see the whole document  --	1-64
A	EP, A2, 0272098 (CITY OF HOPE NATIONAL MEDICAL CENTER) 22 June 1988 see the whole document  -- -----	1-64

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. PCT/EP 89/00981

SA 30725

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EIDP file on 28/02/90.  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0329822	30/08/89	NONE		
WO-A1- 88/10315	29/12/88	NONE		
EP-A1- 0310229	05/04/89	AU-D- 2318188 WO-A- 8901050		01/03/89 09/02/89
EP-A2- 0303155	15/02/89	DE-A- 3726934 JP-A- 1071500		23/02/89 16/03/89
EP-A2- 0272098	22/06/88	NONE		